PLAQUE REDUCTION USED TO DETERMINE SERUM TITERS OF CALVES INFECTED WITH KERATO CONJUNCTIVITIS

by

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INTRODUCTION

Detection and estimation of virus specific antibodies have always been an important procedure in experimental medicine and virology. Estimation of the immunological response stimulated by an antigen is also a measure of its antigenicity.

Virus neutralization test constitutes the basic serologic procedure for study of viruses. Rendering the infectious viral particle noninfectious by specific antibody forms the basis of virus neutralization. Its high degree of immunological specificity makes it the standard against which other serological methods are usually evaluated (Schmidt and Lennette, 1965).

With the introduction into animal virology of the technique of plaqueing of viruses on monolayer cell cultures by Dulbecco (1952) an accurate in vitro method of quantitation became practical.

A plaque is an area of cellular degeneration and death initiated by a single viral particle. Therefore, the number of plaques produced by a viral suspension bears a linear relation to the number of virals particles or plaque forming units in the suspension.

An application of the plaque technique for virus-neutralization test or plaque reduction neutralization test is considered the "most accurate measurement of virus neutralizing capacity of test serums," (Schmidt and Lennette, 1965).

Infectious kerato conjunctivitis is a disease of cattle that has plagued the cattle industry for years. The disease causes huge monetary losses in feed lot cattle by loss of weight, milk and partial loss of

sight. Amidst conflicting reports and diverse organisms labelled as etiological agents of the disease, a filterable agent was isolated by Anthony (1963) from feed lot cattle in Kansas. He reproduced the disease in experimental animals, recovered the virus on tissue culture and studied the cytopathic effects (CPE) produced by the virus and identified the virus as similar to the Infectious Bovine Rhinotracheitis virus.

The objective of the present study is to develop a plaque assay and plaque reduction neutralization test for this virus using serums collected from calves that have been inoculated with the virus.

REVIEW OF LITERATURE

Plaque Assay

Biological assay of viruses is based on absolute numbers of virus units to produce specific response in groups of populations of test hosts (Cunningham, 1966).

Following the demonstrations by Enders, Wellers and Rubin (1949) that viruses could be grown readily on cells outside the animal body, a quantitative plaque assay for viruses utilizing cultured cells was introduced by Dulbecco (1952). He developed the plaque assay on the bacteriophage model. The first animal virus studied by him using plaque assay on tissue culture cells was Western Equine Encephalitis virus.

Grube (1963) observed that plaque development under an agar overlay on infected monolayers thus confining cytopathic effects (CPE) to plaques "is recognized as the most sensitive and accurate method of assay of virus infectivity quantitation and neutralization."

The plaque assay for titration of animal viruses has been universally recognized. Merchant et al., (1965) stated that it was precise and permitted isolation of clones of viruses possessing distinctive biological properties and was more amenable to statistical and genetic analyses of virus cell interactions than any other assay method.

Lunia, et al., (1951) found that the plaque method of titration was equally accurate when compared with the results obtained with electron microscopic enumeration of bacteriophage particles.

Cunningham (1966) described titration of viral infectivity as the enumerative response, being assessed from focal lesions produced by a live virus particle.

Dulbecco (1952) proved that each plaque was formed by a single complete infective virus particle and that the number of plaques formed bore a linear relation to the number of infective particles in a given suspension. The number of plaques produced in a given suspension was the function of the dilution, and plaque counts followed a linear curve and "were consistent with Poisson distribution."

Since its development by Dulbecco (1952) the technique has been used by many investigators for quantitative studies on animal viruses. Stinebaugh and Melnick (1962) while studying plaque formation by Simian Virus 40 (SV_{40}) observed that, at the end point, the cytopathic effect (CPE) was variable and limited to a portion of the culture. They suggested that a good deal of time must be spent meticulously examining the cultures under the microscope. They further observed that cultures would degenerate nonspecifically if they were stored for several weeks which complicated the results.

Dulbecco (1952) used chicken embryo fibroblasts in Petri dishes and incubated the cultures under carbon dioxide.

Cooper (1955) used Tris buffer (hydroxymethyl aminomethane) to control pH and eliminated the necessity of CO_2 incubation. He developed a plaque assay for animal viruses by using agar suspension of cells instead of monolayers.

Black and Melnick (1955) studied plaque formation in Herpes B virus and reported that plaques were formed when the inoculum was small.

Hopkins et al. (1966) found that the plaques development of African horse sickness virus was better achieved when 0.1 ml of inoculum was used than when 0.2 ml was used.

Youngner (1956) studied the dynamics of adsorption and plaque formation of three strains of poliomyelitis, herpes B, and vaccinia viruses. He found that with poliomyelitis virus, the adsorbed as well as diffused particles produced plaques, whereas with herpes virus only adsorbed particles produced plaques. Sabin (1957) used the plaque technique for the selection of attenuated clones of poliomyelitis virus. He was able to achieve a high order of reduction in neurovirulence in vaccine strains. Porterfield (1959) studied plaque production of yellow fever virus and related arbor viruses.

Hsuing and Melnick (1957) plaqued poliomyelitis, cosackie and orphan viruses in bottle cultures of monkey kidney cells in monolayer incubated under CO_2 pressure. They simplified Dulbecco's method by substituting common flat bottles with rubber stoppers. The tight stoppers were found to control pH shifts. They found the plaque method to be more sensitive than the tube method using the microscopic CPE end point.

Dulbecco (1952) and Granoff (1959) plaqued New Castle Disease virus on chick embryo fibroblast culture. Rapp et al. (1959) used immuno-fluorescent technique for enumeration of infectious particles of measles virus. Frothingham and Granoff (1961) measured virus infectivity of mumps virus on chick embryo cultures in petri dishes.

The plaque technique is a useful tool in diagnostic virology. Plaques of different viruses show characteristic patterns and these are utilized for identification. Hsuing (1962) used differential cell culture and

the plaque technique to identify viruses. Distinctive plaque patterns of enteroviruses and different host cell ranges were taken advantage of to identify the agents just as selective media in diagnostic bacteriology.

Rhim and Melnick (1961) investigated the failure of Rheo viruses to form plaques and were able to get precise virus assay by using a serum free overlay. Sheinin (1961) found that fetal calf serum which is extremely critical for ox, horse, and calf cells was unsuitable for polyoma virus plaques.

Grube (1963) assayed virus infectivity and plaque reduction using parainfluenza viruses and immune serum and found the method to be sensitive. Melnick et al., (1965) designed a quantitative experiment using a plaque assay for counting cells infected with herpes zoster virus. They reported that the appearance of plaques on a monolayer depended on the latent period. Stuf and Bang (1966) plaqued mouse hepatitis virus (MHV-2) on primary macrophage cell cultures (30899). They used 30 ml plastic bottles closed with siliconized stoppers, Chang's medium, Difco Noble agar overlay, and neutral red for staining.

Merchant et al., (1965) summarized the factors involved in the successful use of the plaque assay for titration of animal viruses.

1. Hardiness and stability of the susceptible tissue culture cell line. The criterion of stability is the ability of the cells to survive in the maintenance medium long enough to permit lysis of cells by virus used. The cells should be intact and surviving after addition of the overlay.

2. Inherent cytopathic affects of the virus will decide whether plaque titration will be applicable.

3. Young log phase cells at their physiological optimum should be used when they form a monolayer.

4. pH control is critical within the permissible ranges. A suitable buffer system usually helps to control shifts in the pH. When critical pH conditions are essential and large scale work is contemplated an incubator with a humidified atmosphere of 95% air and 5% CO_2 is necessary.

5. A very important consideration is the composition of the maintenance medium. It should be such that it should provide adequate nutrition to the cells to keep them in optimum physiological status. Susceptibility of mammalian cells to viral infection is influenced by their physiological status.

6. The agar overlay may sometimes inhibit plaque formation. The possible presence of toxic materials in the overlay of agar must be recognized.

The Tissue Culture Cells

The technique of cultivating animal cells on solid surface using a fluid medium was originated and developed by Earle and his coworkers Gey and Gey, 1936; Earle, 1943; Hanks, 1948; Hanks and Wallace, 1949; and Dulbecco and Vogt, 1954. They observed that most primary dispersed tissue cells and a wide spectrum of established cell lines grew this way. Abbercrombie *et al.* (1954) observed that they follow a logarthmic growth rate in 24 hours and in 4-9 days monolayers were formed. Due to contact inhibition they form monolayers without piling up. This method was reported by Dulbecco (1952) to be reproducible and facilitated day to day observation of the cells forming into a monolayer, on glass or plastic surface. Tissue culture cells were of two types: I) primary and II) continuous line. Primary cells were directly derived from the tissues of the host animal. These cells were subcultivated for a limited number of passages. A continuous cell line was capable of indefinite cultivation provided adequate nutrition is provided and the cells not allowed to overpopulate. The cells become adapted to *in vitro* conditions, developed

polyploidy and finally stabilized with the unusual number of chromosomes (Prier, 1966). Madin and Darby (1958) reported the establishment of a stable fetal bovine kidney cell line. These cells support the growth of 23 viruses (Warren and Cutchins, 1957).

Merchant et al., (1965) reported that these cells were fibroblastic in type, resembling fibroblasts seen in vivo. They had nothing in common with fibroblasts of living tissue. These cells in tissue culture were spindle-shaped and grow in a loose net work. They described that "a relatively clear cytoplasm a juxta nuclear area known to consist of the Golgi apparatus and vacuoles marks the fibroblast cell from tissue culture cell and also a large, oval, translucent centrally located nucleus."

Media

Eagle (1955) determined the basic requirements for monolayer cell growth. Prier (1966) quoted Eagle (1955-59) described essential and nonessential requirements of amino acids for cell growth. The amino acid requirement of cells differed based on their nutritional selectivity and the number of viable cells in culture. Excess amino acids will inhibit a growth and low levels did not support growth. The carbohydrate required by most cells was glucose in a concentration of 0.1-0.4 per cent. Thiamine, biotin, riboflavin, choline, folic acid, nicotinamide, pyridoxol and pantothenic acid were the basic vitamins required for cultivated cells. The essential ions required for tissue cultured cells were sodium, potassium, magnesium, calcium, chloride and phosphate. Various basal salt solutions were devised to meet specific requirements of cell cultures,

(Earle, 1943; Hanks and Wallace, 1949; Dulbecco and Vogt, 1954.) Prier (1966) stated that cells in culture required factors found in serum and in other naturally occurring substances for their growth and that the most common biological additive was mammalian serum. He observed that serum protein was important in the primary attachment of cells in monolayers and for multiplication. In routine laboratory use, serums are inactivated at 56 C for 30 minutes to remove viral inhibitors or toxic fractions that may injure cells or inhibit virus. Prier (1966) further observed growth factors such as insulin and protamine are found to stimulate growth of He La cells.

Chemically Defined Media

Earle (1943), Hanks and Wallace (1949) and others devised basal salt solutions (BSS) which were essentially similar but slightly different in composition. Many varieties of media were constructed on these basal salt solutions by different laboratories to suit particular cell types. These required only serum (free of inhibitors) as a biological additive. Advantages are claimed that these media are very useful in any assay system.

Schmidt and Lennette (1965) observed that Eagles' minimum essential medium (MEM) was considered to contain optimal quantities of nutrients for in vitro cells. Its composition was based on results of extensive studies on nutritional needs of mammalian cells. It contained 13 amino acids, 9 vitamins and glucose in either Earles or Hanks BSS. Schmidt and Lennette (1965) further reported that this medium would maintain cell cultures for a relatively long time and with serum permit growth and

multiplication of cells.

Overlay Medium

Tytell and Newman (1963) found that agars used for overlay contained acidic polysaccharides which were plaque inhibitors. They plaqued herpes simplex virus on an agar free overlay to overcome plaque inhibitors in agar medium. Liebahar and Takemato (1961) showed that a sulfate polysaccharide in agar inhibited plaque formation and affected the morphology of encephalomyocarditis (EMC) virus plaque and that the inhibitor was released in the process of sterilization. Dextran, a polybasic water soluble ultramolecule was able to allow larger mutant plaques to form from r^+ small plaques on a plaque assay of EMC virus with DEAE (diethylaminoethyl cellulose) overlay. They found that protamine sulphate produced a similar effect.

Brown and Packer (1964) experienced the same inhibitory effect of agar overlay while plaqueing WEE virus. They overcame the inhibitory effect of agar by the addition of protamine sulfate to the overlay medium.

Hopkins et al. (1966) reported that purified Difco agar did not develop plaque inhibitory substances during sterilization. Washing Special Noble agar 20 times in distilled water removed the inhibitor.

With certain herpes viruses such as herpes simplex, inhibitors in agar prevented maximum development of plaques. Even addition of protamine sulfate failed to yield maximum plaque formation. A substitute medium composed of methyl cellulose in a nutrient solution increased the sensitivity of the assay ten fold (Tytell, 1963). Schulze and

Schleisinger (1963) studied plaque formation using dengue virus 2 and a methyl cellulose overlay.

Tytell (1963) observed that the standard plaque technique depended on an agar overlay to prevent released virus from traveling from one cell to another. The agar overlay however did not prevent short distance travel to neighboring cells so that infected centers are set up which lead to plaque formation. The addition of immune serum to the overlay resulted in neutralization of virus released into the medium so that plaques could form only if virus could pass directly from cell to cell without coming into contact with antibodies. Plaques could form without an agar overlay provided the antiserum was added to the nutrient fluid. He reported that herpes virus reacted in this manner. Black and Melnick (1955) found that plaques were formed without agar overlay if the medium contained specific immune serum. Domok and Simon (1966) studied the inhibitory effects of agar extracts instead of agar, on plaques of ECHO virus. They suggested that agar contained two polysaccharides- agaropectine and agarose. Agarose allowed free migration of histones by electrophoresis whereas basic proteins were strongly adsorbed in normal agar, and was free of negatively charged ionized groups. It was used in a concentration of 1.5 per cent for plaqueing.

Domok and Simon (1964) further reported that agar extract (as recommended by Schulze and Schleisinger, 1963) in the overlay inhibited plaque formation by ECHO virus type 19. They also found that calf serum in the medium reduced the virus titer. They used a starch gel overlay as recommended by DeMaeyer and Schonne (1964) to produce plaques.

Staining for Plaque Identification

Stevens and Groman (1963) used crystal violet as described by Holland and MacLaren for staining plaques of infectious bovine rhinobracheitis virus. Hopkins et al. (1966) used neutral red for staining plaques of African horse sickness virus. Merchant et al. (1965) recommended staining with Wright's Stain after removing methyl cellulose overlay for staining plaques.

Plaque Reduction Neutralization

Dulbecco and Vogt (1956) suggested that with the advent of the plaque reduction technique of animal viruses, it was possible to follow the course of neutralization reaction in an adequate manner. Schmidt and Lennette (1965) described virus neutralization test by plaque reduction and observed that a plaque reduction of 80% to be significant.

Kalter (1957), DeSomer and Prinze (1957), and Farrell and Reid (1959) modified the technic by inoculating a virus concentration calculated to produce confluent plaques, then added agar overlay and placed on the agar surface paper discs impregnated with immune serum. The neutralizing capacity of the serum was determined by its ability to inhibit infection of the cells around the disc. Wecker (1960) used immune serum incorporated into the overlays at various concentrations and virus strains inoculated to the cell sheets. The ability of some strains to overcome serum dilutions and produce plaques on monolayers was taken as the criterion for strain differentiation. Dewan et al. (1963) impregnated virus onto paper discs and placed them on the agar

surface of an overlay containing immune serum. Heterologous viral strains were recognized by their ability to produce plaques at higher serum concentrations. Haye and Chame (1966) studied antigenic variation among polyoma virus strains by the plaque reduction neutralization test.

Based on electron microscopic studies, Samuel Dales (1965), discussed the morphologic aspects of and quantitative data of cell virus interaction under the influence of immune serum. They observed that antibodies caused clumping of a large proportion of viral particles in suspension. Neutralized virus became attached to the cell surface and later some were drawn into vacuoles and when a low serum dilution was used. In dilutions of 1:20 and higher were used, subsequent stages of viral replications occurred. The result of interaction of virus with antibody was summarized by them as:

1. Fewer particles of neutralized virus become cell attached.

2. Virus release from phagocytic vesicles become blocked.

3. Treatment with antibody renders (vaccinia) virus susceptible to total degradation within the vacuole, whereas virus treated with preimmune serum releases virus particles which infect other cells adjacent to form a plaque.

Lafferty (1963) discussed the kinetics of "Interaction with Virus and Antibody." He observed that when viruses interacted with their antibodies a complex series of reactions occurred resulting in

1. Formation of a freely reversible combination between virus and antibody.

2. This reaction is followed by a reaction that leads to the formation of a more stable combination between virus and antibody.

Lafferty (1963) also stated that not all the virus particles in a reaction mixture were inactivated at the same rate by neutralizing antibody. This was shown to be due to at least in part to the protection of a fraction of virus particles in the reaction mixture from inactivation by neutralizing antibody.

MATERIALS AND METHODS

Tissue Culture Cells

Fetal bovine kidney cells (FBK) were used. The cells were received from a commercial company* in 1 ml quantities suspended in 99 ml of Hanks growth medium. The cells were centrifuged at 600 r.p.m. for 3 minutes in a Sorvall** anglehead centrifuge. The supernatant was decanted and the cells were suspended in 200 ml Eagles medium containing 10 per cent lamb serum. The media also contained 200 units of penicillin, 200 ug streptomycin and fungizone 2 mg per liter of media. The suspension of cells was thoroughly mixed by drawing into a pipette and vigorously expelling 3 or 4 times for even distribution. The suspension was then distributed into milk dilution bottles (10 ml), Falcon plastic bottles (4 ml), or roller tubes (1 ml). After tightly replacing the stoppers or screw caps the bottles or tubes were incubated at 37 C. After keeping them motionless for 48 hours the bottles and tubes were examined daily until monolayers were established. The medium was changed when the indicator changed color due to a shift in pH of the medium.

As monolayers were established in milk dilution bottles they were utilized as cell seed cultures and were transferred. Two ml of growth medium were added to each milk dilution bottle and the cell sheets were scraped gently with a sterile rubber policeman. The scrapings were pooled in a sterile flask containing the required quantity of growth medium.

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*Colorado Serum Company, Denver, Colorado.

**Ivan Sorvall Inc., Norwalk, Conn.

The suspension was vigorously mixed with a 10 ml pipette with a large bore orifice and seeded into bottles. The rate of transfer adopted was 1:3 for milk dilution bottle. Monolayers in plastic bottles were used for experimental work. Trypsin dispersal or dispersing with Salneversene and trypsin was also done to establish monolayers.

Medium

Eagles minimum essential medium (MeM) with 10% lamb serum was the standard growth medium. The growth medium used contained Earle's basal salts 1% (E.B.S.) and MEM amino acid 2% (50x), MEM vitamin solution 1%, l-glutamine 1%, and 10% lamb serum. This medium was reported to permit longer periods of culture of mammalian cells without refeeding (Eagle, 1959).

The basic salt solution and amino acids were mixed and the pH was adjusted with 1N NaOH or 7.5% $NaHCO_3$ to 7.2±. The medium was completed by the addition of vitamins, antibiotics, and lamb serum. The serum was inactivated by heating to 56 C for 30 minutes, and the medium was sterilized by filtration. Glutamine was added to the medium as a final step just before use due to its instability. The medium was stored at 4 C for not more than one week. Maintenance medium was identical to growth medium except it did not contain serum. All chemicals used were of reagent grade. Media were tested for sterility in nutrient broth.

Virus

The virus used in the experiments was obtained from Anthony (1963). The virus has been isolated from lacrymal secretions of calves into clinical signs of infectious bovine kerato conjunctivitis. The virus had been identified as Infectious Bovine Rhinotractreitis virus. The virus strain had been sealed in 5 ml glass ampoules and stored at -60 C.

Agar Overlay

The Agar overlay was prepared by dissolving two grams of purified Difco* agar or special noble agar Difco or Nutrient Agar Bacto in 100 ml of double distilled water. The pH of the water was adjusted to 7.0. The agar solution was autoclaved for 20 minutes at 10 lbs pressure/sq. inch. The sterilized agar solution was dispensed while hot into sterile screw capped tubes, and stored at 4 C. At the time of use the agar was melted in a steamer or boiling water bath.

The nutrient medium used was a double concentration of maintenance medium with 5% inactivated lamb serum.

The melted agar and medium were held separately at 45 C for 30 minutes in a water bath. The nutrient medium and the agar solution were mixed in equal quantities and held at 45 C until used. This was the standard agar overlay and will be referred to as first agar overlay.

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*Difco Laboratories, Michigan.

Second Overlay

The overlay medium for staining was the same as described above but contained Neutral red* solution at the rate of 1 ml of a 1% solution for 100 ml of medium. Inactivated lamb serum was added to the medium at the rate of 2.5%.

Methyl Cellulose Overlay

Methyl cellulose** (M.C.) was repeatedly washed with absolute alcohol and ether and was air dried as described by Rapp et al. (1959). Two grams of the dried methyl cellulose powder was suspended in 50 ml double distilled water at 100 C under vigorous shaking and was autoclaved for 20 minutes and cooled to 45 C. Equal quantities of M.C. and double concentration maintenance medium containing 5% serum were mixed and cooled to 4 C. The M.C. solution dissolved completely at 4 C. The M.C. stock solution was stored at -20 C. When used it was brought to 4 C.

The staining of bottles with M.C. was done by one of two methods. The bottles were either stained with Wright's stain or standard agar overlay containing neutral red.

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*National Amline Div., New York, New York.

**Fisher Scientific Products, New Jersey.

Protamine Sulfate

Five-tenths of a gram of protamine sulphate* was suspended in 10 ml of maintenance medium, stirred and warmed in a water bath for 30 minutes and then autoclaved at 10 lbs for 20 minutes. The substance does not dissolve completely but the suspension remains viscous. This salt was incorporated in the overlay medium in a concentration of 0.5 mg/ml of medium.

Hydrocortisone

Prednisolone** acetate was dissolved in maintenance medium and was used to pre-treat the monolayers before infecting them. The drug was used in a final concentration of 0.0625 mg/ml.

Inoculation

Tenfold dilutions of the virus were made in maintenance medium. Monolayer cultures were washed with maintenance medium after draining the bottles of the growth medium and 0.1 ml of virus dilution was placed on the cell sheet. This quantity of inoculum was used in the experiments. After replacing the screw caps tightly the bottles were incubated at 37 C for one hour. During incubation the bottles were rocked gently at 15 minute intervals to enhance even distribution of the virus on the cell sheet. All virus dilutions were made in maintenance medium.

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*City Chemical Corp., New York, New York.

**Schering Corp., New Jersey.

Serums

Three rabbits were inoculated with the stock virus to obtain hyperimmune serum for plaque reduction tests. One milliliter of the virus was injected subcutaneously followed by three more intravenous-injections of one ml each at weekly intervals. The rabbits were bled one week after the last injection, and after separation, the serums were stored at -20 C until used. The serums were handled under sterile conditions. All serums were inactivated at 56 C for 30 minutes before use. The virus used for inoculating the rabbits for preparation of immune serums was obtained from the original stock culture.

The bovine immune serums used in this study were previously collected by Gray and Anthony (1966). These serums were collected from calves that had been experimentally inoculated with the identical virus strain used in this work.

Serums were identified, catalogued and stored at -20 C.

Controls for each procedure consisted of a cell control and virus control. The cell control bottles were not inoculated with virus and the virus controls did not receive serum dilutions. Virus controls showed CPE in 36 hours (Plate I, Appendix).

EXPERIMENTAL PROCEDURES AND RESULTS

Plaque Assay with Purified Agar Difco

Experiment I. Fetal bovine kidney cells (FBK) cultures were prepared in 30 ml plastic bottles. When confluent sheets of cells formed, the growth medium was drained off and the cultures were washed two times with five ml of maintenance medium. The cell cultures were inoculated and incubated for 1 hour at 37 C to facilitate adsorption of the virus.

A nutrient agar overlay as described earlier was prepared by mixing equal quantities of double concentration of Eagles maintenance medium in Earles BSS with five per cent filter sterilized inactivated lamb serum and two per cent previously melted Difco purified agar held at 45 C.

After incubation for one hour the unadsorbed virus suspension was drained off. Four ml overlay medium was added to each bottle culture. They were kept in an upright position till the agar cooled and just began to flake. At this point the bottles were laid flat on the table for 30 minutes. After the agar overlay had hardened the bottles were incubated at 37 C for 48 hours.

At the end of 48 hours the bottles were removed from the incubator and a second overlay with neutral red was prepared and 4 ml were added to each bottle and were further incubated at 37 C for 8 hours.

The bottles were then removed from the incubator and the plaques were counted under a colony counter. The bottles were held in the refrigerator for 48 hours and plaques again counted. This was used as the standardized method for enumerating plaques.

The average plaque counts obtained after 3 assays were shown in Table 1. The plaques in dilution 10^{-4} to 10^{-5} were confluent and could not be counted. The counts of dilutions 10^{-6} through 10^{-8} were counted. No plaques were observed at a 10^{-9} dilution. Cell controls did not have plaques and virus control for CPE in 10^{-6} and 10^{-7} had cytopathic effect. An average plaque count of 209 in 10^{-6}, 83 in 10^{-7} and 11 in 10^{-8} dilutions of virus were obtained with purified Difco Agar overlay.

Table 1. Enumerated plaque counts obtained under the different conditions of the experiments.

Experiment Number	Virus Dilutions 10^{-6}	10^{-7}	10^{-8}	
	Plaque Counts			
I	209	83	11	Agar overlay with serum.*
II	236	98	9	Methyl cellulose with serum.**
III	221	93	7	Agar overlay with serum. Ninety minutes incubation.
IV	214	96	8	Serum free overlay--agar.

*Purified agar Difco.

**Methyl cellulose 100 centipoises.

Plaque Assay with Methyl Cellulose Overlay

Experiment II. One-tenth ml of the virus dilutions 10^{-4} through 10^{-8} were used to infect monolayers. After incubation each bottle was overlaid with 4 ml of methylcellulose with 5% serum and incubated 48 hours. After 48 hours the second agar overlay containing neutral red was added

to one set of bottles and to a second set the methylcellulose was loosened with Earles basal salt solution and removed. These bottles were stained with Wright's stain and plaques were enumerated. The Wright stained plaque counts were 229, 100 and 212 in 10^{-6}, 10^{-7} and 10^{-8} dilutions of virus. The second set of bottles stained with neutral red had counts of 236, 98 and 9 on 10^{-6}, 10^{-7} and 10^{-8} dilutions. Plaques appeared round or oval and small. They had irregular margins under 10x magnification. Plaques were more easily observed with the methylcellulose overlay.

Plaque Assay with Purified Agar Overlay with Prolonged Incubation

Experiment III. Monolayer cell cultures were infected with 0.1 ml of virus dilutions 10^{-4} to 10^{-8}. The dilutions were made in maintenance medium as before. One-tenth ml of each viral dilution was inoculated into identified bottles and incubated for 90 minutes. After incubation the unadsorbed virus suspension was decanted and 4 ml of agar overlay was added and incubated for a further period of 48 hours. The second overlay containing neutral red was then added. After a further incubation of 8 hours plaques were enumerated under the colony counter. Inoculated bottle cultures were incubated for 90 minutes instead of one hour to investigate whether prolonged incubation would have any effect on plaque counts due to better adsorption. Results of plaque counts indicated that there was no better adsorption of virus in one and one-half hours than in one hour. Plaque counts of 221, 93 and 7 were obtained in 10^{-6}, 10^{-7} and 10^{-8} respectively (Table 1).

Serum Free Overlay with Purified Difco Agar

Experiment IV. The standard plaque technique was modified as the agar overlays did not contain serum. Identical virus dilutions and incubation periods were used as described for the standard method. The exact duplicate of the procedure was run 48 hours later.

Plaques obtained had a better definition. Plaque counts in the two experiments did not vary from the counts made when serum was incorporated in the overlay. Counts of 139 and 214 in 10^{-6}, 74 and 96 in 10^{-7}, and 10 and 8 in 10^{-8} dilutions of virus.

Effect of Protamine Sulfate on Plaque Size

Experiment V. Protamine sulfate in a concentration of 0.5 mg/ml was incorporated in the overlay medium. Standard virus inoculations and incubation periods were followed. Plaque size was somewhat increased when protamine sulphate was used in the overlay. Plaque numbers were not increased. Counts of 130 and 127 in 10^{-6}, 72 and 65 in 10^{-7} and 6 and 8 in 10^{-8} were obtained. Controls with standardized overlay had 180 plaques in 10^{-6} dilution, 81 in 10^{-7} and 10 in 10^{-8}.

Effect of Prednisolone Acetate

Experiment VI. Monolayers were treated with prednisolone acetate in a concentration of 0.0625 mg/ml for 20 minutes. The prednisolone solution was decanted and 0.1 ml of virus dilution was added and incubated for one hour. At the end of one hour methyl cellulose overlay was laid on the monolayer and incubated for 72 hours. An agar overlay

containing neutral red was overlaid at this period. The plaques enlarged and coalesced by this time and the cells began to disintegrate. About 160 small plaques could be counted in 24 hours. The bottle cultures were inoculated with 10^{-6} virus dilutions.

Plaque Reduction Neutralization

Experiment VII. Fetal bovine kidney cell monolayers in plastic bottles were washed twice with maintenance medium. Serum-virus mixtures were incubated at 4 C for 1 hour. After the incubation of the serum-virus mixtures, two-tenths of a ml of each of the mixtures were inoculated onto monolayers and incubated for 1 hour at 37 C.

The test serums were inactivated for 30 minutes at 56 C and 1:2, 1:4 and 1:8 dilutions of the test serums in maintenance medium were made and identified. Serums collected from rabbits that had received four injections of virus were also included in plaques reduction test.

The virus which was previously titrated was diluted in maintenance medium. Tenfold dilutions were made so that, at the appropriate dilution (1:800,000) 0.1 ml contained 400 TCID/50.

The serum-virus mixtures were prepared by adding equal volume of virus dilution to each tube containing an equal volume of the serum dilution. The mixture contained a constant concentration of virus and varying concentration of serum.

The agar overlay was then added to each bottle after draining off the unadsorbed mixture, as was done for plaque assay. The bottles were then incubated at 37 C for 48 hours.

At the end of this period the second agar overlay containing neutral red was added and incubated 8 hours at 37 C. Plaques were then enumerated using the colony counter.

Serums collected from calves two days post inoculation failed to reduce plaques (Table 2). Eight day post inoculation the serums were capable of neutralizing viruses as plaque counts were reduced. By 10 to 14 days serums contained more neutralizing antibodies as there was an average of 60% plaque reduction. Plaque reduction counts were most significant in serums collected 60 days post infection. Some neutralizing antibodies were present in serum collected from calf 444 one hundred and five days post inoculation. The results of plaque reduction neutralization on 41 serums are presented in Table 2. Rabbit hyperimmune serum reduced plaques 90 per cent.

Table 2. Plaque reduction obtained with pre and post inoculation serums from experimental infected calves.

Calf Number	Day	Serum Dilutions 1:2	1:4	1:8	Day	1:2	1:4	1:8
		Plaque Counts				Plaque Counts		
401	0	18	18	42	2	9	22	34
404	0	17	19	35	3	11	23	29
408	0	15	19	41	8	4	15	27
434	0	26	30	38	10	6	14	27
407	0	27	27	43	10	4	10	23
433	0	24	38	39	13	2	9	28
465	0	25	34	44	14	3	10	22
405	0	16	18	32	14	1	6	19
460	0	21	26	41	15	7	13	27
440	0	9	16	37	18	3	12	21
406	0	16	18	40	21	5	8	14
428	0	24	27	39	60	3	6	19
429	0	24	26	45	60	0	9	12
430	0	19	28	41	60	2	11	14
444	0	20	26	36	105	6	14	17

DISCUSSION

Plaque assay on the kerato conjunctivitis virus has not been reported. For each animal virus, plaque assay systems have different sensitivities. A preliminary study of the different conditions of experiment had to be determined to find the conditions which would give satisfactory efficiency of plating. When this was determined the plaque reduction test was designed.

In preliminary trials with Bacto agar and special agar-Noble the plaque assay was not satisfactory. With Bacto-agar, plaque formation was not clear. With special Noble agar faint plaques became oriented but were not well defined, were few in number, and faded.

Tytell and Newman (1963) reported that inhibitors in agar affected plaque formation. The same effect was found with unwashed special Noble agar. With purified agar the efficiency of plating improved.

Overlays with purified agar Difco combined with equal parts of Eagles Medium were added to infected cell cultures one hour after incubation. Staining with neutral red after 68 hours incubation was used as the standardized plaque identification technique. The composition of the overlay was modified to assess the effect of such modifications on the assay system compared with the standardized plaque technique. Hopkins et al. (1966) reported that virus dilutions made in maintenance medium gave a higher PFU titre than dilution in phosphate buffered saline without calcium and magnesium (PBS^{-}). Virus dilutions in all the experiments in this work were made in Eagles maintenance medium. The PFU titres obtained were more uniform.

Exclusion of serum from the overlay medium gave better definition to the plaques. Lamb serum was used in the plaque assay of the kerato conjunctivitis virus. In the plaque assays conducted without serum the PFU titres did not significantly differ from the assay conducted with serum included in the overlay medium.

Bottle cultures which were overlaid with methylcellulose and stained by Wright's method, were examined microscopically by 10x. The number of plaques were in near agreement with the PFU titres obtained by the corresponding bottle cultures under standardized technique. The addition of protamine sulfate to the overlay was reported by Liebaher and Takemoto (1961) to have overcome the inhibitory effects of sulfate polysaccarides released during sterilization of agar. Hopkins et al. (1966) observed protamine sulphate and reported that they obtained an increase in plaque diameters of African horse sickness virus. Results obtained with the use of protamine sulfate in the overlay were in agreement with the results obtained by Hopkins et al. as the plaque size was slightly enlarged.

The effect of treatment with predisolone acetate in a concentration of 0.0625 mg/ml before infecting with virus was studied. Plaques appeared early during the incubation period, rapidly enlarged and the cell monolayer disintegrated in seventy-two hours.

In one experiment conducted the standardized technique was used except for 90 minutes incubation. It was found that longer incubation did not effect PFU numbers.

The results of reduction tests on test serums revealed that at 2 days post inoculation plaque reduction was not evident. Eight days

post inoculation there was some plaque reduction. Ten to fourteen day post inoculation serums had an average of 60% neutralizing capacity. A significant feature was that 60 days post inoculation serums had most significant reduction. In calf 444 there was some evidence of neutralizing antibodies one hundred and five days post infection. Rabbit hyperimmune serum significantly reduced plaques by 90 per cent.

SUMMARY

A plaque assay technic was developed for kerato conjunctivitis virus for the performance of plaque reduction tests on serums collected from experimental calves inoculated with infectious bovine kerato conjunctivities virus. Fetal bovine kidney cells were used for the cell-virus system. Of the different conditions of experiment used to obtain maximum efficiency of plating, purified Difco agar overlay gave the most satisfactory results.

Two per cent purified Difco agar was used as an overlay with double concentration of Eagles maintenance medium, containing 5% serum. This was added after incubating infected monolayers for one hour at 37 C. Bottle cultures were further incubated for 48 hours and a second agar overlay containing neutral red and 2.5% serum was added and plaques counted after 8 hours incubation. This was the standardized plaque assay procedure followed. Exclusion of serum from the overlay medium did not effect plaque counts.

Addition of protamine sulfate to the overlay in a concentration of 0.5 mg/ml resulted in a slight increase in plaque size. Prednisolone in a concentration of 0.0625 mg/ml was used to pretreat monolayers before infection. This caused small plaques to appear in 24 hours but further incubation for 72 hours caused rapid disintegration of monolayers. Incubation for one and one-half hours after infection of cells did not enhance the plaque count.

Eagles minimum essential medium in Earle's BSS containing 10% lamb serum was the standard growth medium and the same without serum was the maintenance medium.

Plaque reduction tests were conducted by the standardized plaque technic. The inoculum consisted of equal quantities of serum and virus mixture containing constant concentration of virus and varying concentration of serum. Serum-virus mixtures were incubated for one hour at 4 C and inoculated on the cell sheets. The rest of the procedure was the same as in standard plaque technic. The plaque counts obtained on different test sera were compared against the count on bottles with virus alone. The plaque reducing capacity of the serums at various periods post inoculation were determined.

Plaque reduction test on test serums on experimental calves showed that at 2 days post inoculation the serums did not have any neutralizing ability. Antiserums 8 days post inoculation had some evidence of neutralizing antibodies. Ten to fourteen days post inoculation serums had an average of 60% plaque reduction. The most significant plaque reduction occurred with serums collected 60 days post inoculation. Some evidence of plaque reduction was observed in serum of calf No. 444 one hundred and five days post infection. Rabbit hyperimmune serum reduced plaques by 90 per cent.

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APPENDIX

EXPLANATION OF PLATE I

Fig. 1. Fetal bovine kidney cells--uninfected; X40.

Fig. 2. Fetal bovine kidney cells--showing cytopathic effects 48 hours after inoculation with kerato conjunctivitis virus; X96.

PLATE I

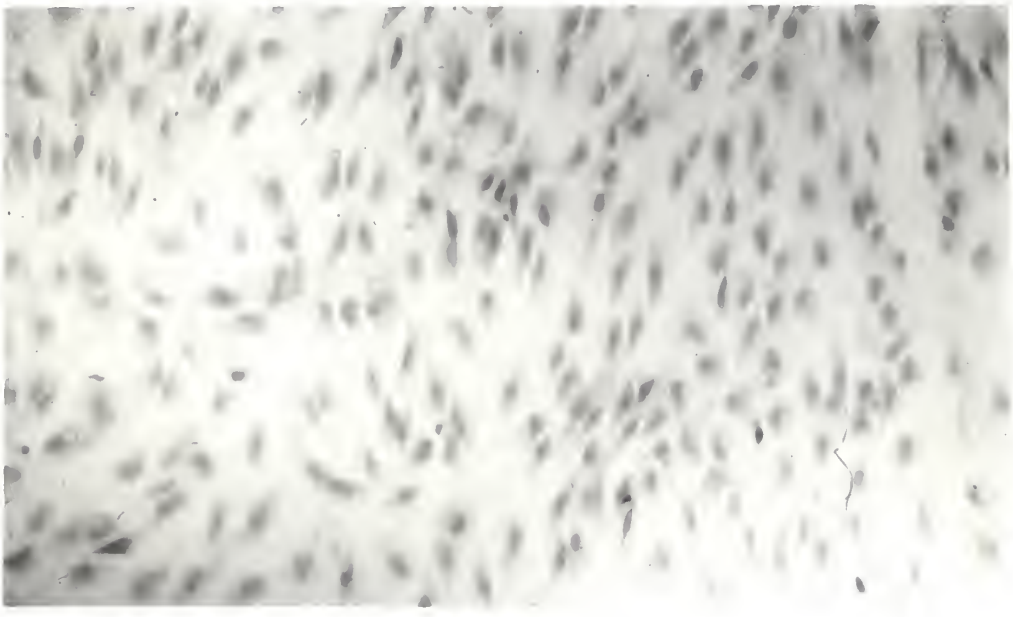

Fig. 1

Fig. 2

PLAQUE REDUCTION USED TO DETERMINE SERUM TITERS OF CALVES INFECTED WITH KERATO CONJUNCTIVITIS

by

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

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1967

A plaque assay technic was developed for infectious bovine kerato conjunctivitis virus, for determining titers of serums from experimental calves inoculated with the identical strain of the virus. Fetal bovine kidney cells in monolayers were used for the cell virus system.

Different overlay media were used to determine the most suitable for obtaining efficiency of plating. Purified Difco agar gave the most satisfactory results.

The standardized technic consisted of infecting washed cell monolayers with 0.1 ml of virus dilution in maintenance medium, incubating for one hour and adding an agar overlay consisting of equal quantities of double concentration of Eagles maintenance medium, containing 5% filter sterilized inactivated lamb serum and two per cent purified Difco agar held at 45 C. After further incubation for 48 hours at 37 C a second agar overlay medium containing neutral red and 2.5% serum was added. Plaque counts were read after 8 hours incubation. Eagles minimum essential medium with Earles Basal Salt solution containing 10% lamb serum was the standard growth medium and the maintenance medium contained no serum. The pH was kept at 7.2.

Addition of protamine sulfate in a concentration of 0.5 mg/ml to the agar overlay medium resulted in a slight increase in plaque size. Pretreatment of monolayers with prednisolone in a final concentration of 0.0625 mg/ml caused appearance of small plaques in 24 hours, but with further incubation plaques rapidly enlarged and coalesced, disintegrating the monolayer at 72 hours.

Prolonged incubation for one and one-half hour did not affect plaque counts. Serum free agar overlay did not alter the plaques or counts.

Plaque reduction neutralization tests were done using the standardized plaque technic. Serum virus mixtures were made in Eagles maintenance medium pH 7.2, so that the mixtures contained equal quantities of a constant concentration of virus and varying concentration of test serums. The mixtures were allowed to react for one hour at 4 C. The rest of the procedure was the same as in plaque assay. The capacity of test serums to reduce the plaque forming units titer of the virus was determined by comparing plaque reduction induced by serums that were collected at different intervals post inoculation.

The results of plaque reduction tests showed that serums collected from calves two days post inoculation failed to reduce plaques. Eight days post inoculation the serums had antibodies as indicated by neutralization of some of the virus shown by reduction of plaque counts. By the tenth to fourteenth day serums contained neutralizing ability and plaques were reduced by an average of 60%. Plaque reduction counts were most significant in serums collected 60 days post infection. Some neutralizing antibodies were present in serum collected from calf 105 days post inoculation.